BIODIVERSITY AND PHYLOGENETIC STUDIES OF LAND SNAILS (PULMONATA: STYLOMMATOPHORA) IN PENINSULAR MALAYSIA

By

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

LIST OF ABBREAVIATIONS

bp	Basepair
DNA	Deoxyribonucleic acid
rRNA	Ribosomal ribonucleic acid
LSU	Large subunit
ITS	Internal transcribed spacer
dNTP	Dinucleotide triphosphate
EDTA	Ethylenediamine tetra-acetic acid
CTAB	Cetyl trimethylammonium bromide
HCl	Hydrochloric acid
NaCl	Sodium chloride
TE	Tris-EDTA
NaAC	Sodium acetate
$MgCl_2$	Magnesium chloride
g	Gram
kb	Kilobase
L	Litre
M	Molar
mm	Milimetre
mM	Milimolar
rpm	Rotation per minute
U	Unit
µM	Micromolar
µL	Microlitre

KAJIAN BIODIVERSITI DAN FILOGENETIK SIPUT DARAT (PULMONATA: STYLOMMATOPHORA) DI SEMENANJUNG MALAYSIA

ABSTRAK

Sejumlah 24 spesies dari 21 genus dalam 14 famili yang disampel dari 19 lokasi di Semenanjung Malaysia telah dikenalpasti dengan melihat pada ciri morfologi cengkerang dan dibandingkan dengan spesimen baucar di Natural History Museum, London. Kesemua spesimen berjaya dijujuk berdasarkan pengamplifikasian berganda gen rRNA yang terdiri daripada kawasan konservatif dan variabel (gen 5.8S, kawasan ITS-2 lengkap dan subunit besar gen 28S). Jujukan taksa dari Semenanjung Malaysia ini dianalisis bersama 179 data Stylommatophora yang tersedia ada berdasarkan jujukan 844 bp untuk mengkaji hubungan filogenetik mereka. Kedua-dua pohon filogeni, Neighbour-Joining (NJ) dan Maximum Likelihood (ML) kekal monofiletik (seperti kajian terdahulu), disokong dengan pemisahan kepada klad “achatinoid” dan “non-achatinoid” (40% dan 100% nilai bootstrap NJ dan ML, masing-masing) termasuk yang dari Malaysia. Klad “Achatinoid” mempunyai empat kelompok yang stabil dan ketara, iaitu, (1) Plectopylidae, (2) Streptaxidae dan Diapheridae (Streptaxoidea), (3) Ferussaciidae, and (4) lineaj yang mengandungi Subulinidae, Achatinidae, Coeliaxidae dan Thyrophorellidae. Daripada tujuh kumpulan utama “non-achatinoid”, status taksonomi bagi Helicoidea, Orthalicoidea, Elasmognatha dan Orthurethra, kekal tidak dapat diselesai kerana tiada takson tambahan atau hanya satu wakil takson yang diperoleh dalam kajian ini. Namun begitu, kumpulan di dalam takson Limacoidea, Clausilioidea dan Arionoidea memerlukan kajian lanjut yang mungkin menjurus

kepada semakan taksonomi. Kajian ini menonjolkan kepentingan penyelidikan struktur dalaman yang mendalam untuk resolusi taksonomi yang tepat dalam kajian di masa hadapan. Khususnya, status polifiletik *Macrochlamys* spp. dan beberapa spesies lain mungkin disebabkan ketidaktentuan bersandar hanya kepada ciri morfologi luaran. Penemuan ini juga merupakan data pertama yang meluas untuk Stylommatophora Malaysia, terutamanya daripada famili Ariophantidae.

BIODIVERSITY AND PHYLOGENETIC STUDIES OF LAND SNAILS (PULMONATA: STYLOMMATOPHORA) IN PENINSULAR MALAYSIA

ABSTRACT

A total of 24 species from 21 genera in 14 families sampled from 19 locations in Peninsular Malaysia were identified by looking at their shell morphological characteristics and compared with voucher specimens in Natural History Museum, London. All the specimens were successfully sequenced based on the double amplification of rRNA genes that comprised of the conserved and variable regions (5.8S gene, the complete ITS-2 region and the large subunit 28S gene). These Malaysian taxa sequences were then analyzed together with available 179 Stylommatophoran data based on 844 bp sequences to investigate their phylogenetic relationships. Both Neighbour-Joining (NJ) and Maximum Likelihood (ML) phylogenetic trees remained monophyletically, supported (as in previous analysis) with the splitting into 'achatinoid' and 'non-achatinoid' clades (40% and 100% in NJ and ML bootstrap values, respectively) with the Malaysian inclusion. The 'achatinoid' clade had four distinct and stable clusters' namely (1) Plectopylidae, (2) Streptaxidae and Diapheridae (Streptaxoidea), (3) Ferussaciidae, and (4) a lineage that includes Subulinidae, Achatinidae, Coeliaxidae and Thyrophorellidae. From the seven major groups in the 'non-achatinoid' clade, the taxonomic status of Helicoidea, Orthalicoidea, Elasmognatha and Orthurethra remained unresolved as no additional or only a single representative taxon was obtained in the present study. However, groups within the Limacoidea, Clausilioidea and Arionoidea taxa require further investigations that may lead to taxonomic revisions. The study highlights the importance of a comprehensive investigation of the internal structure for precise

taxonomic resolution in future studies. Specifically, the polyphyletic status of *Macrochlamys* spp. and several other species may be attributed to the ambiguities in relying only on external morphological characteristics. The findings are also the first extensive data on the molecular taxonomy of Stylommatophora in Malaysia, the Ariophantidae in particular.

CHAPTER 1

Introduction

1.1 General introduction

Snails from the class Gastropoda in the phylum Mollusca are typically recognized in having a muscular foot, a mantle overlaying the internal organ along with a single coiled shell which protects the entire or parts of the body (Burch and Pearce, 1990). The subclass Pulmonata consisting the land snails or slugs are hermaphrodite and that includes with two pairs of tentacles. The group also includes several freshwater and marine, in addition to terrestrial families (Berry, 1963). Land snails have high distributions in limestone area or soil with high calcium carbonate (Schilthuizen *et al*., 2003) for the reason that they require calcium for their shell development together with egg production (Berry, 1963; Kerney and Cameron, 1994).

The order Stylommatophora is regarded as the most successful terrestrial pulmonates (Dayrat *et al*., 2011) and have broad distribution in isolated islands, mountains in the southern hemisphere as well as tropical forests (Barker, 2001) with 92 known recognized families (Emberton *et al*., 1990). Until recently, the Stylommatophoran classification has been mainly based according to morphological characteristics alone, which in general have been efficient at species level. However, at the deeper level (family) their relationships and phylogeny remains complicated (Wade and Mordan, 2000). Molecular studies are now increasingly utilized to address taxonomic questions.

In many instances, molecular markers can address evolutionary relationships where the morphological characteristics are unable to provide precise information (Wade and Mordan, 2000; Sutcharit *et al*., 2010; de Weerd and Gittenberger, 2013). Recognizing the efficiency of the rRNA region that comprised of conserve and variable parts (5.8S gene, the complete ITS-2 region and the large subunit 28S gene), Wade and Mordan (2000) utilized this marker to successfully elucidate evolutionary relationships over a wide range of taxa within this group. Wade *et al*. (2001; 2006) postulated the Stylommatophoran pulmonate was split into two distinct clades, which they referred as 'achatinoid' and 'non-achatinoid'. The basal placement of the 'achatinoid' clade was not easy to explain on morphological characteristics (Mordan and Wade, 2008).

But to date, nearly all molecular studies on the pulmonate gastropods have been focused on a limited number of taxa (Dinapoli and Klussmann-Kolb, 2010; Holznagel *et al*., 2010; Dayrat *et al*., 2011). Thus, despite the continuous studies being conducted with additional taxa with the utilizion of rRNA gene cluster (Wade and Mordan, 2000; Wade *et al*., 2001; 2006), the phylogeny and evolution of the Stylommatophoran pulmonates are still unclear at the "deeper familial" level. However, the phylogenetic resolution at a higher level of superfamilial and infraordinal groups have been successful with high bootstrap supports for Achatinoidea, Helicoidea, Limacoidea, Clausilioidea, Orthurethra and Elasmognatha (Mordan and Wade, 2008).

Taxonomic knowledge of a majority of the snail species in Malaysia is rather poor (Maasen, 2001) and up to date revision is very limited (Choh *et al*., 2006). More

recent studies in Malaysia have emphasized on the diversity and distribution of land snails especially in the limestone karts and forest reserve, population studies, systematics revision and species description (Schilthuizen *et al.*, 2003, 2005; Liew *et al.*, 2008; Schilthuizen and Liew, 2008; Liew *et al.*, 2009; Hoekstra and Schilthuizen, 2011). Thus, the overall aim of this study is to further elucidate the understanding of the phylogenetic relationships of Stylommatophoran pulmonates based on the combination of the conserved and variable regions of rRNA gene cluster. Specifically, it will provide insights into the evolution of Stylommatophoran pulmonates in Peninsular Malaysia and its relationships with existing data.

1.2 Objectives of the study

1. To determine the diversity of Stylommatophoran pulmonates in Peninsular Malaysia
2. To construct the molecular phylogeny of Malaysian Stylommatophoran covering a wide range of taxa
3. To construct a comprehensive phylogenetic relationships (tree) among the Stylommatophora through additional taxa to the existing data

CHAPTER 2

Literature Review

2.1 The Class Gastropoda

Class Gastropoda is a large taxonomic group commonly known as slugs or snails, belonging to animal phylum Mollusca with an estimate of approximately 35,000 known living species (Solem, 1984; van Bruggen, 1995). The range of size, shape and form in molluscs are immerse, but gastropods can generally be distinguished from other animals by possessing a well developed muscular foot, a mantle covering the internal organ and a single coiled shell which covers all or parts of the body (Burch and Pearce, 1990; Kerney and Cameron, 1994).

This class can be further divided into three subclasses; Opistobranch, Prosobranch and Pulmonata (Hickman *et al.*, 2006). Opistobranch are marine and bisexual snails. The shell is usually absent or reduced. Two pairs of tentacles are present. On the other hand, Prosobranch are dioecious snails i.e. having two distinct sexes in separate individuals and can be found on land and freshwater but most of them are marine snails. Prosobranch can be easily distinguished by having an operculum, a calcareous plate that is horny, carried on the dorsal surface of the foot when the snail is crawling (Figure 2.1). This plate will seal the shell aperture when the snail withdraws inside the shell for protection (Schepman, 1908). The Pulmonata is the air breathing land snail or slug, having a “lung” in the mantle cavity and is equipped with two pairs of tentacles (Berry, 1963). It is a hermaphrodite animal, with both male and female reproductive organs in the same individual but the union of two individuals is required during mating (Tryon *et al*., 1885).

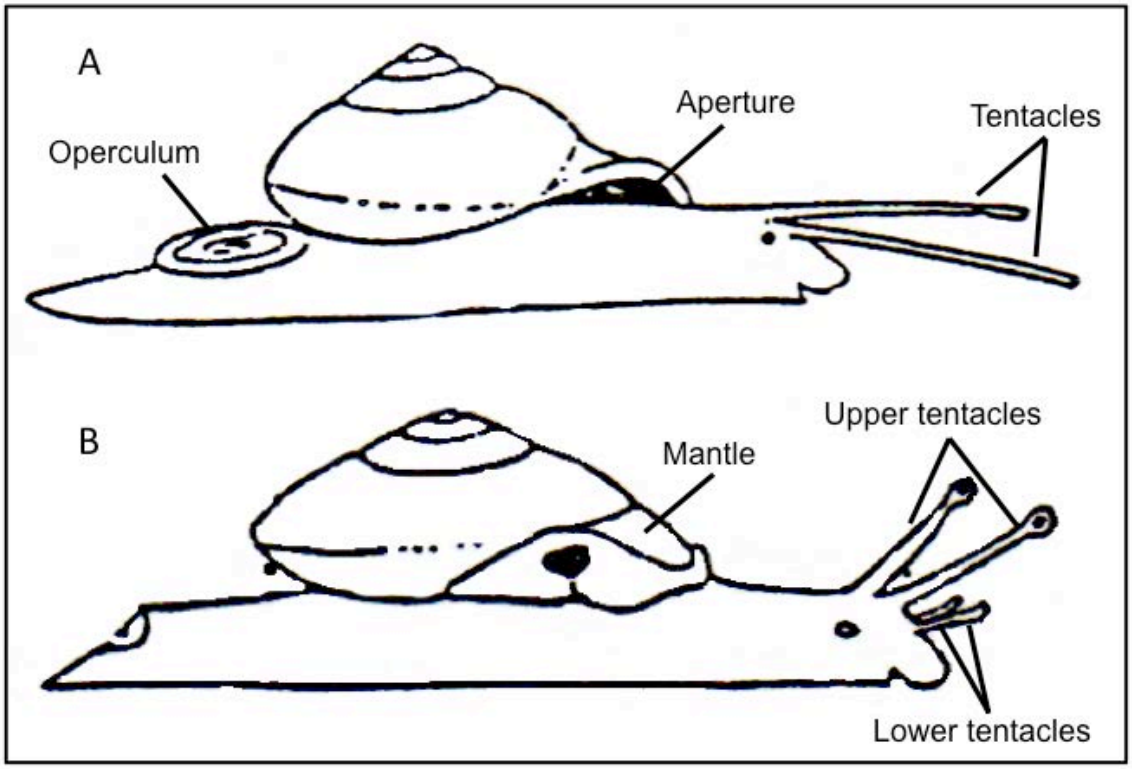

Figure 2.1: (A) Prosobranch snail (B); Pulmonate snail (modified from Berry, 1963).

Land snails are easyly recognized by their spirally coiled shell (Naggs *et al.*, 2006). The shell characteristics such as colour, shape, size and coiling direction are the important keys for species identification (Figure 2.2) (Burch and Pearce, 1990). A slug is a land snail without the external shell (Figure 2.3). In snail, the body remains in the shell whereas a slug's mantle only covers part of the front body (Kerney and Cameron, 1994). The absence of shell permits speedier movement of the slugs and the ability to enter small crevices where the environment is high in moisture (Barker, 2001).

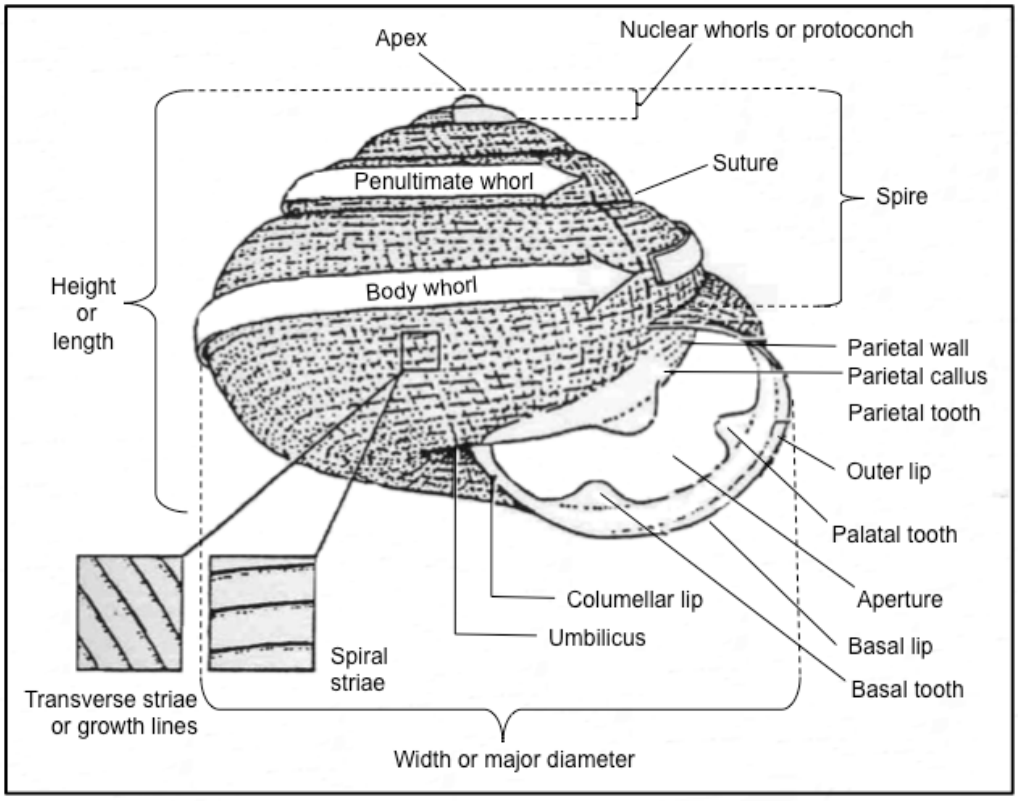

Figure 2.2: A general diagrammatic representation of a land snail shell (modified after Burch and Pearce, 1990).

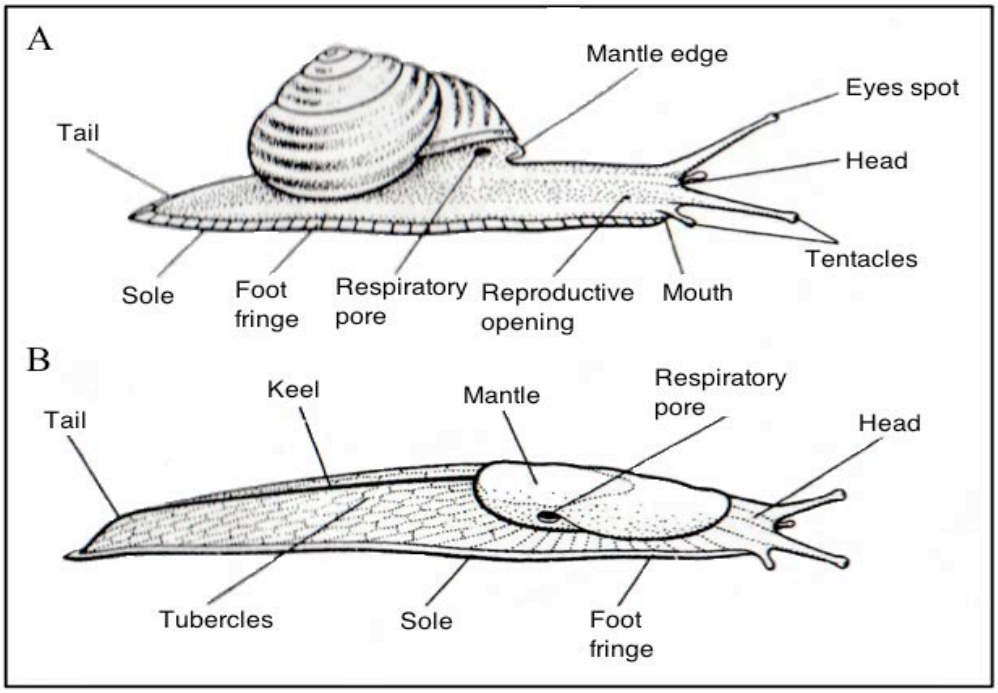

Figure 2.3: External features (A) Land snail; (B) Land slug (modified from Kerney and Cameron, 1994).

Most snails and slugs are detritivore, feeding on decaying materials. Some are pests, eating growing plants causing damage and loss of high value crops. Snails that live on damp rocky surfaces, scrape for lichens and algae as food. There are also carnivorous snails, e.g: the family Streptaxidae, preying on other snail species, earthworms and small invertebrates (Berry, 1963; Srivastava, 1992; Iglesias and Castillejo, 1999).

Most of the terrestrial snails are oviparous, lay eggs with no embryonic development within the mother (Heller, 2001). Many are ground dwelling species, deposit their eggs under the leaf litter, on the soil surface or under the soil. Arboreal species which living on trees, usually deposit their eggs in humus or between branches so that water can be collected at the base for maximum moisture (Barker, 2001; Heller, 2001). Egg cannibalism also takes place in several stylommatophoran species (Heller, 2001) e.g: *Zonitoides arboreus* scatter their eggs in the forest litter to avoid cannibalism (Baur, 1994).

Land snails require calcium for their shell growth as well as egg production (Berry, 1963; Kerney and Cameron, 1994). Thus, this accounts for their high distributions in limestone areas such as limestone karsts, soils from limestone or soil with high calcium carbonate, which provide their calcium supply (Schilthuizen *et al*., 2003). They can absorb the calcium directly or by consuming plant, water, calcium carbonate from soil content, leftout shells or bird faeces on the ground (Johannessen and Solhøy, 2001).

2.2 General Overview on Stylommatophora Pulmonates

The order Stylommatophora is the most successful terrestrial pulmonates (Dayrat *et al*., 2011). They are widely distributed in isolated islands, mountains in the southern hemisphere as well as tropical forests (Barker, 2001) with 92 known recognized families (Emberton *et al*., 1990). Dayrat and Tillier (2002) defined Stylommatophora based on three synapomorphies: the ability to retract their tentacles, existence of a membrane covering the pedal gland and secondary ureter. They could be the first pulmonate that appeared during the Upper Carboniferous (300 Ma) (Dayrat *et al*., 2011).

Since they are known to have different types of dietery behaviour (most are detritivores, while the rest are herbivores and carnivores), the radula is an essential characteristic for systematics and taxonomic identification at the generic and species level (Barker, 2001). Stylommatophoran are hermaphrodites, producing both oocytes and spermatozoa in one individual. Thus self-fertilization is common but the frequency varies among the species. Figure 2.4 shows the general reproductive system of Stylommatophoran snails.

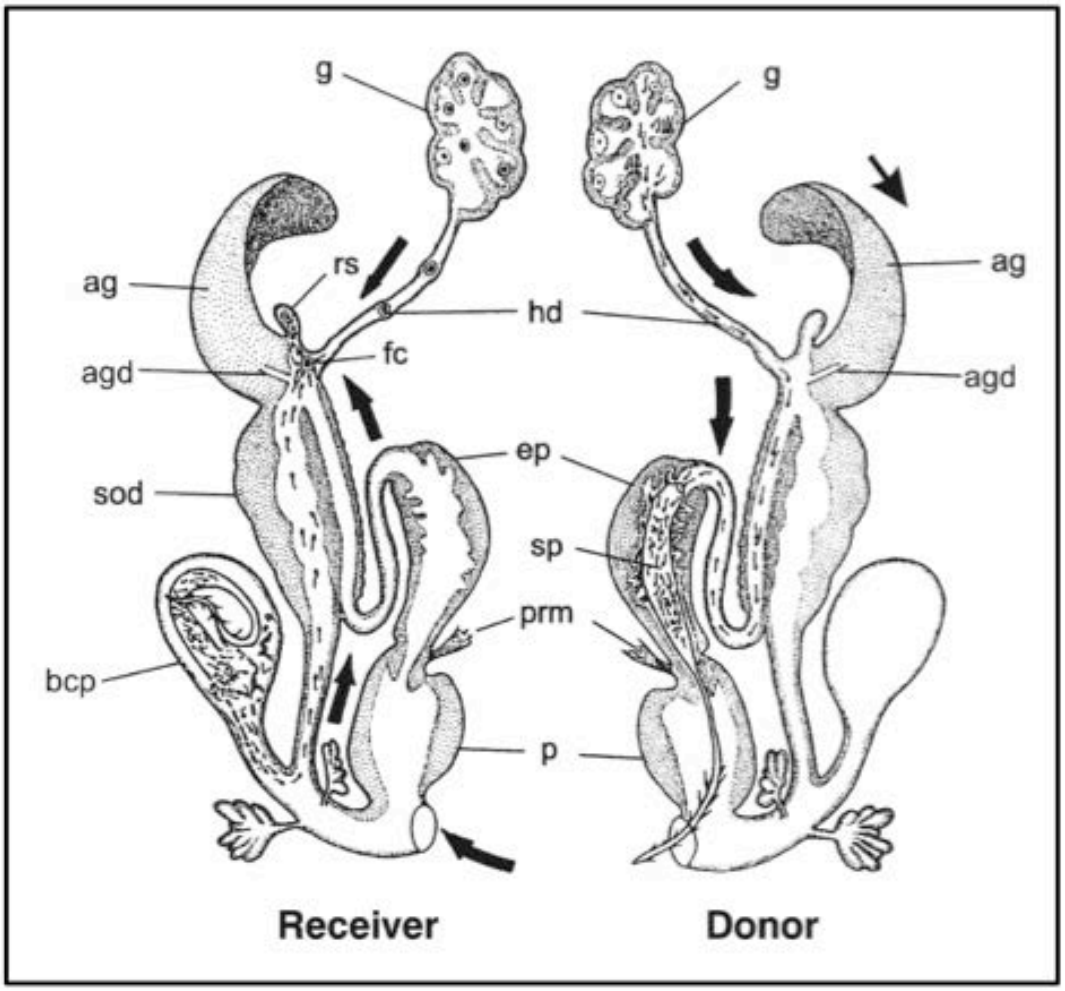

Figure 2.4: The reproductive system of a Stylommatophoran. ag: albumen gland; agd: albumen gland duct; bcp: bursa copulatrix; ep: epiphallus; fc: fertilization chamber; g: gonad; hd: hermaphrodite duct; p: penis; prm: penis retractor muscle; rs: receptaculum seminis; sod: spermoviduct; sp: spermatophore; vs: vesicular seminalis (modified after Gomez, 2001).

At the family level, the most primitive Stylommatophoran excretory system is sigmurethrous (‘sigmoid’ ureter). The mesurethran (no ureter), heterurethran (ureter is at the front of kidney margin) and orthurethan (straight ureter) clades are believed to have derived from it (Wade *et al*., 2001). In an initial phylogenetic investigation, the Orthurethra group is the most advanced Stylommatophoran by its equivalent position with other recently evolved superfamilies. However, in a later study with additional taxa (160 species, 144 genera, 60 families), the Stylommatophora was shown to cluster into two clades; the ‘achatinoid’ and ‘non-achatinoid’ (Wade *et al*., 2006). They defined the ‘achatinoid’ clade by the absence of fully developed stimulator, which regarded as autapomorphy of this group while the ‘non-achatinoid’ have fully developed stimulator in many of its major group with no autapomorphy identified. Based on the current distribution of the Gondwanan groups (the acavoids *s.l* in Africa, Madagascar, the Seychelles, Sri Lanka, Australia and South America), the superfamilial clades in Stylommatophoran is hypothesized to have diversified during the Mesozoic period.

2.3 The Importance of Land Snails

Land snails have varied life modes which play different ecological roles. The understanding of how they react with different types of animals and their habitat is significant when it affects the human economy and health (Berry, 1963). Being small, slow moving, high endemism and low dispersal capabilities, land snail is an efficient and important model to understand the mechanism of evolution (Davison, 2002). By understanding the distribution of organisms; it is easy to reconstruct the historical biogeography especially when using immobile organisms. This is because their distribution patterns are expected to be more informative for palaeogeographic

reconstructions. Thus, the slow moving animal like land snail is the best organism to study historical biogeography (Hausdorf, 2000). Reconstruction of the paleoenvironment from the fossil of land snail shells can help us to understand the isotope stability of H and N, periodic growth lines and pedogenic carbonate deposition on the shells (Goodfriend, 1992).

Snails can be of medical importance when they act as secondary hosts to parasites infecting humans (Berry, 1963). *Neotricula aperta* is the intermediate host for *Robertsiella kaporensis*, a parasite of rodents, which can also infect human. *Oncomelania hupensis* is the host for *Schistosoma japonicum*, the human parasite in Central and Southern China (Attwood *et al*., 2003). A survey in China revealed the snails *Achatina fulica* and *Pomacea canaliculata* infected with the rat lungworm parasite *Angiostrongulus cantonensis* are being eaten by many people which can consequently lead these people being in high risk to Eosinophilic meningitis disease (Lv *et al*., 2009).

Land snails are sensitive to acidification and environment pollution (Johannessen and Solhoy, 2001). Thus, they are suitable animals to serve as environmental pollution indicators. Passerine birds feed on snails to gain the calcium for eggshell development and also good growth of their nestling skeleton. The reduced snail masses in polluted areas affect growth and cause reproductive problem in birds that rely heavily on snails as calcium sources (Eeva *et al*., 2010). Therefore, high levels of trace metals in snails can disrupt the higher level organism up on the food chain (Fritsch *et al.*, 2011). In their biomonitoring study of the land snail, *Arianta arbustorum*, Berger and Dallinger (1993) discovered different urban areas such as industrial minings and cities have different

concentration of cadmium, lead and zinc even between neighboring populations. The pollutant concentration in snail body will also lead to different genetic variability (Jordaens *et al*., 2006).

Land snails display high allopatric diversity, easy to collect and have the persistence of shells (Clements *et al*., 2008; Liew *et al*., 2008). Among all animals, land snails are one of the distinct taxa inhibiting limestone karst (Schilthuizen *et al*., 2005) since they require calcium for shell growth as well as egg production (Kerney and Cameron, 1994). Thus, they have high distributions in limestone areas, which provide their calcium supply (Schilthuizen *et al*., 2003). The limestone karsts that are isolated from other non-calcareous substrata, restricts gene flow resulting in high level of endemism. Moreover, the acidic podzolic soil surrounding the karsts may promote speciation that create barriers to the dispersal of land snails away from the karsts (Clements *et al*., 2008). Human activities such as rock climbing gives negative impact to the snail density, richness and diversity since these activities are conducted at wooded carbonate cliffs and talus slopes which are important habitats of the snails (McMillan *et al*., 2003).

2.4 Biogeography of Land Snails

Crisci *et al*. (2003) defined biogeography as the study of geographic distribution of a living organism. It can be further divided into ecological biogeography and historical biogeography. Historical biogeography focuses on the taxonomic group's historical biogeographic events, including data from geology, biology and geography (Crisci *et al*., 2006). The main purpose of understanding historical biogeography is to

evaluate how the geographical distribution of organisms has been influenced by both ecological and historical processes (Diniz-Filho *et al*., 2008).

Due to low dispersal rate, land snails tend to distribute into fragmented habitats that could result in geographical isolation and genetic structuring (Arter, 1990), reduced gene flow among populations, consequently leading to different evolutionary histories (Chiba, 1998). Thus, data on their genetic structuring can be utilized for detailed reconstruction of past events (Davison and Chiba, 2006). Furthermore, from their historical biogeography data, one can estimate time of lineage splitting events; speciation rates and determine the processes involved in the population assembly. All these can lead to the understanding of evolutionary mechanisms (Holland and Cowie, 2009). It is important to understand the natural history of the living organism as it provides useful insights into how extinct species deal with the pressure in the past (Dayrat *et al*., 2011).

2.5 Applications of Molecular Marker in Land Snails

Many studies have been documented on the historical biogeography, population structure, phylogeny, taxonomy and systematics, evolutionary relationships and phylogeography and conservation genetics of land snails and slugs, utilizing molecular markers such as nuclear and mitochondrial genes (Goodacre and Wade, 2001; Holland and Hadfield, 2002; Sutcharit *et al*., 2006; Elejalde *et al*., 2008; Trewick and Cowie, 2008; Gomes *et al*., 2010; Hoekstra and Schiltuizen, 2011; Jesse *et al*., 2011; De Weerd and Gittenberger, 2013). The combination of the conserved and variable regions of approximately 1460 nucleotides of rRNA gene cluster comprising the 5.8S gene, the

complete ITS-2 region and the large subunit 28S gene have been utilised to resolve the phylogenetic relationships of the Stylommatophora land snails (Wade and Mordan, 2000). They concluded that the rRNA region is suitable for addressing evolutionary relationships over a wide range of taxa. Their study revealed that the Stylommatophora is monophyletic and suggested the applicability of the non-stylommatophoran pulmonates as potential outgroups related studies. Davison *et al*. (2005) reported the correlation of mating behavior and spire position with phylogenetic relationshops. In their study of mating behavior in 60 genera (28 families) of land snails and slugs (Stylommatophora), they observed that three monophyletic groups (Helicoidea, Limacoidea and Phylomycidae) that always mate face to face were usually the low spired snails. On the other hand, the families that mate by shell mounting were the high spired snails. The latter group usually has variable body symmetry.

In many instances, molecular phylogenetic trees successfully clarified taxonomic ambiguities where morphological characteristics had failed to resolve systematic issues (Sutcharit *et al*., 2010). In their investigations, they deduced that genital characteristics such as penial sheath have little value in recognizing the generic level in Streptaxidae. Penial armature is considered as an apomorphy for Streptaxidae but for the case of *Diaphera* and *Sinoennea*, the absence of penial armature is a plesiomorphy since they diverge at the basal position of the Streptaxoidea clade. Thus, while morphological characteristics failed to recognize higher-level relationships in the Streptaxoidea, they were well resolved through molecular data.

Palaeogeographical events during the Pleistocene epoch have also shaped the distribution and genetic structuring of many land snails. Based on the shell morphology and distribution, *Euhadra peliomphala* (family Bradybaenidae) was split into a few subspecies (Nishi and Sota, 2007). However, genetic data on this species are limited (Hayashi and Chiba, 2008). During the early to middle Pleistocene (2.0-0.14 mya), numerous volcanic activities occurred, resulting in landbridge collapse between the southern Boso area and the mainland of Japan. These events created barriers to *E. peliomphala* population gene flow in the past, dividing them into five distinct clades which corresponded to five regions (Shimizu and Ueshima, 2000). The events also caused interspecific hybridizations between *E. peliomphala* and the parapatric species, *E. brandtii*. They also found the shell morphology are intermediate of both snail species in some individuals in the same area. They hypothesized that *E. brandtii* might have been more widely distributed during the early Pleistocene and invaded Southern Boso via the landbridge which then led to hybridization with *E. peliomphala*. After the disappearance of the landbridge, *E. brandtii* may become extinct in the Southern Boso but the introgressed mtDNA was maintained in the remaining *E. peliomphala* as the consequence of maternal lineage survival from the mitochondrial.

Phylogenetic techniques can be applied to species conservation by providing information on the genetic variation among populations or taxa particularly for endangered species (Harvey and Steers, 1999). Although there may be snail populations with low genetic diversity, it is important to maintain haplotypes present in these populations which are absent in other populations (Ross, 1999). The data on genetic diversity may be useful in management and decision making such as the selection of

sites for predator exclusion fences, directing captive efforts and carrying out translocation in the field (Holland and Hadfield, 2002).

2.6 The Land Snail Studies in Malaysia: Current Status

Most of the studies done on land snails in Malaysia esspecially in Peninsular Malaysia have been conducted by several malacologists such as Berry (1963), Collinge (1902), Laidlaw (1931, 1937, 1963), Sykes (1902, 1903), Tomlin (1938, 1939, 1941), Tweedie (1961), van Benthem-Jutting (1949, 1954, 1960, 1961a, 1961b), Venmans (1957) and von Mollendorff (1891). However, the knowledge of many of the species remains very poor (Maasen, 2001) and recent published studies in the twenty first century are very limited (Choh *et al*., 2006). More recent studies in Malaysia are focused on the diversity and distribution of land snails especially in the limestone karts and forest reserve (Schilthuizen *et al*., 2003; Liew *et al*., 2008), population studies (Schilthuizen *et al*., 2005; Hoekstra and Schilthuizen, 2011), systematics revision (Liew *et al.*, 2009), species description (Schilthuizen and Liew, 2008) and historical biogeography (Razalli, 2011).

As in other regions, in Malaysia land snails are in high abundance in limestone kart areas or any area with high calcium content because of their calcium requirements for shell growth and egg productions (Juričkovà *et al*., 2008). In their study in Sabah, Schilthuizen *et. al* (2003) found numerous of land snail specimens in the Tabin site, a limestone area compared to the Danum site, hills surrounded by dipterocarp forest. However, from their analysis, the total diversity was found to be similar in both sites; Tabin (limestone site) had 49 species while 43 species were from Danum (non-

limestones site). They concluded that the limestone and non-limestone sites have similar diversities of land snails with a few species restricted to limestone area and vice versa. Schilthuizen and Rutjes (2001) also found 61 species of land snails in one km^2 area in Danum, a tropical rainforest in Sabah although the abundance was low in the numbers presumably due to the drought effect in 1997/1998 that led the mortality. The high species composition in rainforest area has strong correlation to the altitudinal factors such as the differences in microclimatic conditions (soil and leaf litter moisture, temperature), resource availability, habitat structure (the plant stems density and canopy height) and habitat configuration (garden area and distance to edge) (Raheem *et al.*, 2008).

Hoekstra and Schilthuizen (2011) found that there was a distinct separation of *Gylotrachela hungerfordiana* (family Vertiginidae) subpopulations of East and West of Titiwangsa Range with genetic distance increasing with geographic distance, using combination of the mitochondrial cytochrome oxidase 1 (COI) gene and the nuclear ribosomal DNA internal transcribed spacer (ITS-1). They postulated that the successive colonization and long distance dispersal of *G. hungerfordiana* across Peninsular Malaysia were attributed to migrating mammals, which carry the snails in their fur as well as the prevailing wind directions during the monsoons.

The phylogeny of the specious genus *Everettia* (family Dyakiidae) in Sabah based on the mitochondrial COI and 16S, and nuclear ITS-1 sequences showed that the taxonomy based on the classical morphology such as the shell surface sculpture and body colour patterns are the best characteristics to identify members of this genus (Liew

et al., 2009). They also found that the mountains in Sabah act as barrier to the dispersal of this genus.

Land snail is a good informative model to understand evolution and biogeography on islands (Solem, 1984; Holland and Cowie, 2009). Norhanis *et al.* (2010) presented the first checklist on micro-land snails from Langkawi Island with a total of 14 species from seven genera and five families. From their survey, seven species were new records for Malaysia while the other seven are unidentified morphospecies. Geological separation of limestone formation might cause the different morphological variations in the family Vertiginidae from Langkawi Islands (Razalli, 2011).

CHAPTER 3

MATERIALS AND METHOD

3.1 Sampling Sites and Sample Collection

The land snails were collected from 19 limestone areas in Peninsular Malaysia during the day (Figure 3.1, Table 3.1). Direct searching method (Ausden, 1996) was used to obtain the snails from suitable habitats such as under stones, in crevices of limestone wall and rocks, and in leaf litter. The live snails were then placed in mesh bags together with wet cotton wools to maintain the humidity and prevent desiccation.

3.2 Specimen Storage and Preservation

The snails were photographed and recorded at three different angles, the apertural view, the basal view and the apical view using a digital camera on a white background (several of the photographs were taken by Harry Taylor from Natural History Museum, London) (Figure 3.2). The photos were used to facilitate the species identification. The specimens were soaked in water with menthol crystal for about half an hour to an hour (depending on the species) to relax the tissue (Pearce and Örstan, 2006). Then, a small part of the foot tissue (*ca.* 25 mm^3) was sliced finely from each specimen and placed in 99% ethanol for DNA genomic extraction. The rest of the specimens were kept in 70% ethanol for preservation.

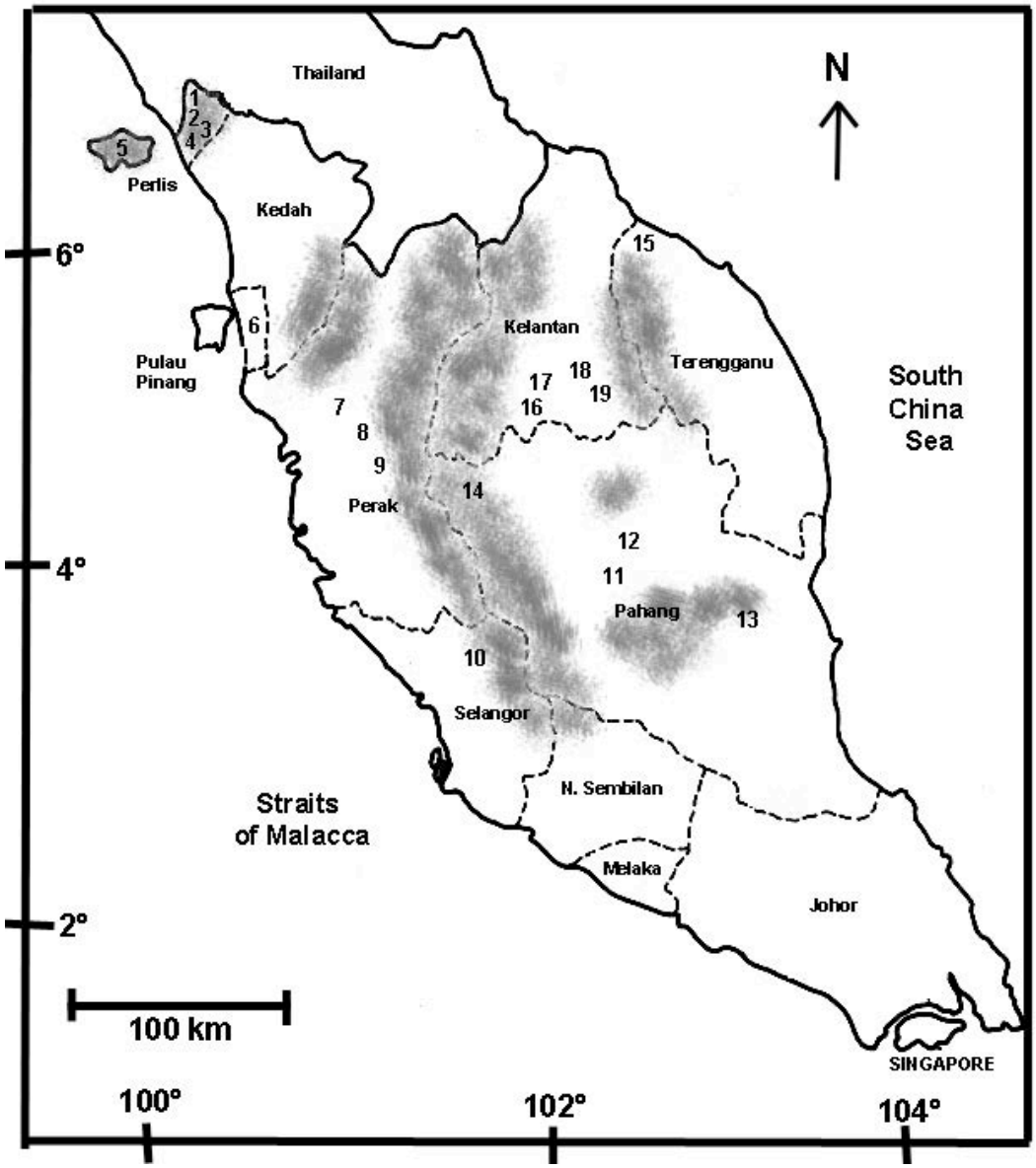

Figure 3.1: Sampling locations of land snails in Peninsular Malaysia. Numbers indicate the study location areas (details in Table 3.1). The light grey shadow in the map indicate the limestone karst.

Table 3.1: Details on the sampling locations, coordinates and date collected. Numbers in bracket indicates location in map in Figure 3.1.

Region	Study Area	Coordinates	Date Collected
Perlis	Wang Burma, Perlis (1)	N 6.7004° - E 100.1871°	April 2009
	Gua Kelam (2)	N 6.6469° - E 100.2002°	April 2009
	Bukit Jerneh (3)	N 6.5483° - E 100.2841°	April 2009
	Wang Belanga (4)	N 6.6812° - E 100.1871°	April 2009
Kedah	Tanjung Rhu, Langkawi (5)	N 6.4415° – E 99.8137°	April 2009
Pulau Pinang	Seberang Perai (6)	N 5.4119° – E 100.5195°	September 2010
Perak	Gunung Lanno (7)	N 4.6096° - E 101.1336°	April 2009
	Gua Tempurung (8)	N 4.4168° – E 101.1878°	April 2009
	Gua Kek Lok Tong (9)	N 4.5549° - E 101.1281°	April 2009
Selangor	Batu Caves (10)	N 3.2371° - E 101.6844°	April 2009
Pahang	Gunung Senyum (11)	N 3.7235° – E 102.4344°	September 2010
	Gua Balai, Kota Gelanggi (12)	N 3.9002° - E 102.4735°	September 2010
	Gua Charas, Bukit Panching (13)	N 3.9054° – E 103.1633°	September 2010
	Cameron Highland (14)	N 4.5069° – E 101.4097°	September 2010
Terengganu	Bukit Keluang, Besut (15)	N 3.8225° – E 101.9915°	September 2010
Kelantan	Pekan Gua Musang (16)	N 4.8840° - E 101.9667°	September 2010
	Bandar Baru Gua Musang (17)	N 4.8569° - E 101.9543°	September 2010
	Gua Bayu (18)	N 5.0939° - E 102.2197°	September 2010
	Perasu (19)	N 4.9189° - E 102.1149°	September 2010

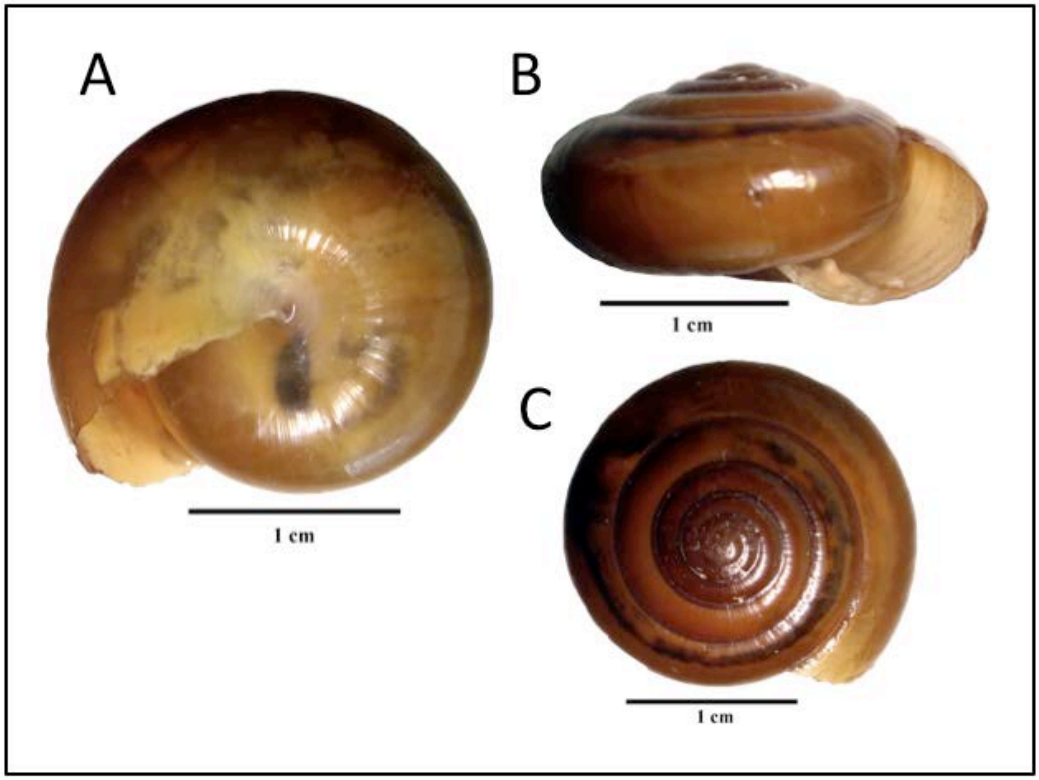

Figure 3.2: Three different angles for snail identification. (A) Basal view; (B) Apertural view; (C) Apical view.

3.3 Species Identification

Samples were prepared by removing the debris using a brush and viewed under a microscope. Identification until genus level was based on shell morphology, according to Laidlaw (1928), van Bentham-Jutting (1961a, 1961b), Berry (1963), Schileyko (2003) and Sutcharit & Panha (2006b) using only key characteristics such as shell shape, coiling direction, terminology of whorls and sutures, types of umbilicus, shell lip and surface markings (Figure 3.3). The shell characteristics were different among species but constant within each species. However, there were individual variations, due to different populations and the age of the snail (Burch and Pearce, 1990). The shell can be differentiated by elongated or depressed shape (Figure 3.4**)**. Coiling direction of the shell can be either sinistral or dextral **(**Figure 3.5**)**. The last whorl is called body whorl, can be well rounded, angular, shouldered or flattened **(**Figure 3.6**)**. Numbers of shell whorls also contribute to the snail identification **(**Figure 3.7**)**. The shell umbilicus can be imperforate, perforate shell, rimately perforate or umbilicate in shape **(**Figure 3.8**)**. The lip or the edge that surrounds the aperture can be expanded, reflected or neither expanded nor reflected lip **(**Figure 3.9**).** Further identification was done by referring to voucher specimens at the Natural History Museum, London by Norhanis Razalli.

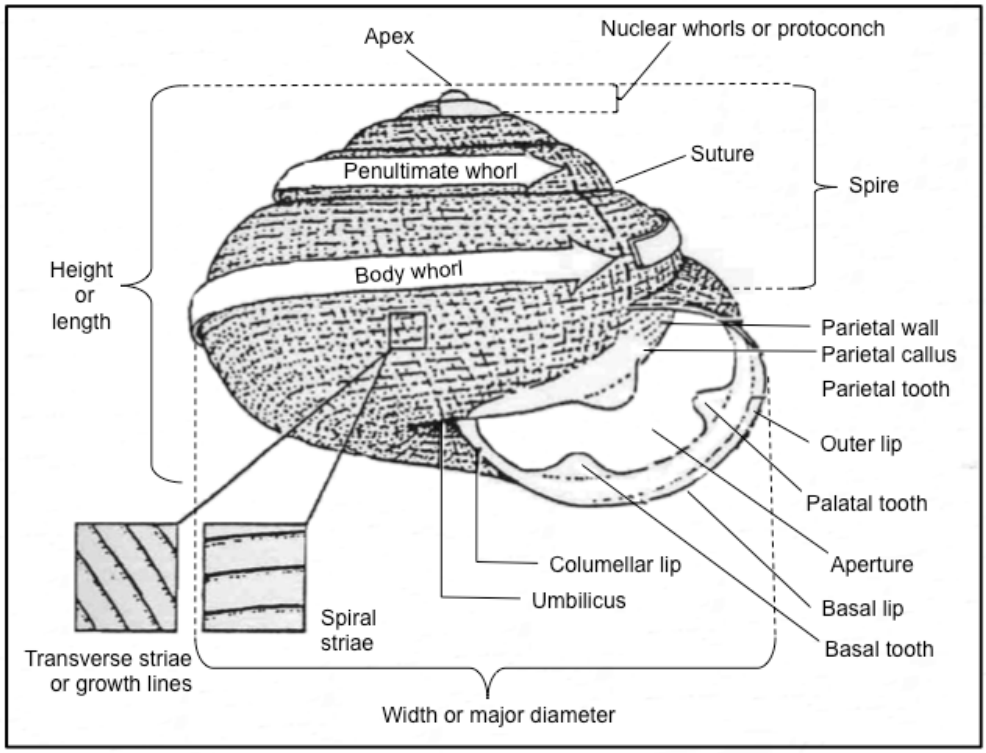

Figure 3.3: Shell morphology for identification (modified from Burch & Pearce, 1990).

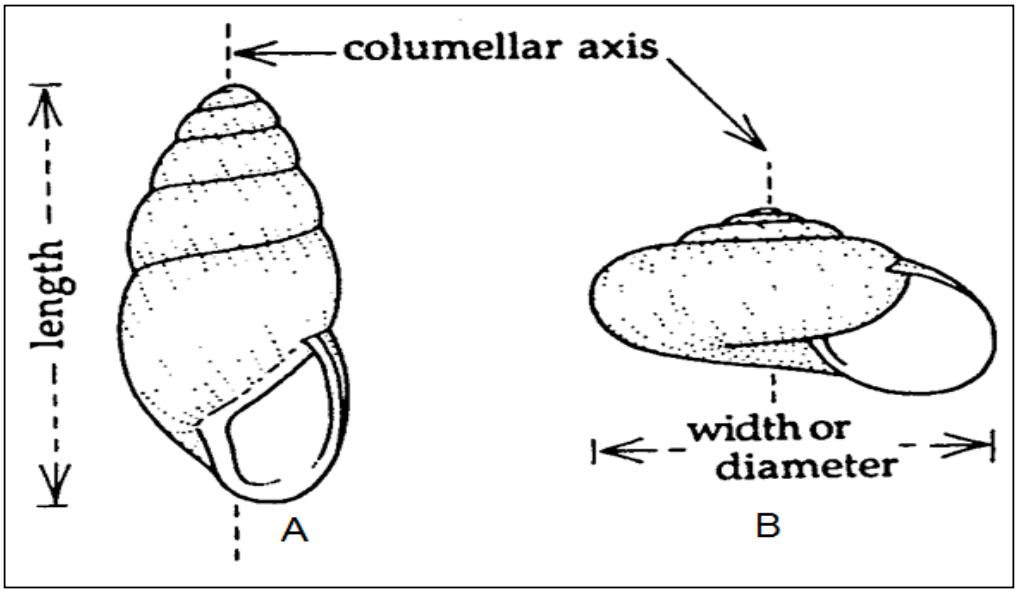

Figure 3.4: Shell shape (A) An elongate shell; (B) A depressed shell (modified from Panha and Burch, 2005).